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## Identification and Characterization of a New Member of the TNF Family that Induces Apoptosis

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### Summary

A novel tumor necrosis factor (TNF) family member has been cloned and characterized. This protein, designated TNF-related apoptosis-inducing ligand (TRAIL), consists of 281 and 291 aa in the human and murine forms, respectively, which share 65% aa identity. TRAIL is a type II membrane protein, whose C-terminal extracellular domain shows clear homology to other TNF family members. TRAIL transcripts are detected in a variety of human tissues, most predominantly in spleen, lung, and prostate. The TRAIL gene is located on chromosome 3 at position 3q26, which is not close to any other known TNF ligand family members. Both full-length cell surface expressed TRAIL and picomolar concentrations of soluble TRAIL rapidly induce apoptosis in a wide variety of transformed cell lines of diverse origin.

### Introduction

Tumor necrosis factor (TNF) is the prototypic member of an emerging family of cytokines that function as prominent mediators of immune regulation and the inflammatory response. The apparent involvement of TNF in septic shock, autoimmune disorders, and graft-versus-host disease is well documented (reviewed by Revel and Schattner, 1987; Cerami and Bautlar, 1988; Cohen, 1988). Eight other members of this family are currently known, including lymphotxin (LT $\alpha$ , TNF $\beta$ ), lymphotxin  $\beta$  (LT $\beta$ ), and ligands for CD40, CD30, CD27, OX40, 4-1BB, and Fas (APO-1) (Cosman, 1994). With one exception, all ligands are type II membrane proteins, with homology confined to the C-terminal ~150 residues. The exception, LT $\alpha$ , appears to be a secreted protein that can also be found cell surface-associated via its interaction with another TNF family member, LT $\beta$  (Browning et al., 1993). In addition, a proteolytically processed soluble form of TNF has long been recognized (Pernica et al., 1984). These ligands interact with a parallel family of some twelve homologous receptors, characterized by cysteine-rich pseudorepeats in the extracellular region, which as with the ligands, are

variably expressed on a variety of cell types, including B cells, T cells, dendritic cells and macrophage (Smith et al., 1994).

Direct evidence for crucial roles of many of these family members comes from aberrant phenotypes arising from spontaneous mutations or targeted gene inactivation in mice. Loss of function mutations in either the murine Fas ligand (*gld*), murine Fas receptor (*lpr*), or human Fas receptor results in lymphadenopathy and autoimmune disorders (Watanabe-Fukunaga et al., 1992; Adachi et al., 1993; Takahashi et al., 1994; Fisher et al., 1995; Rieux-Laucat et al., 1995). One implication is the involvement of Fas in the establishment of peripheral tolerance. Similarly, mutations in human CD40 ligand give rise to hyper-immunoglobulin M (IgM) phenotypes in patients, consistent with in vitro evidence suggesting an essential role in B cell affinity maturation and immunoglobulin isotype switching (Allen et al., 1993). Targeted inactivation mutants for murine TNFR type I (p55) are vastly more susceptible to certain microbial infections, such as *Listeria monocytogenes*, consistent with a protective effect for TNF (Pfeiffer et al., 1993; Rothe et al., 1993). Finally, disruption of the murine LT $\alpha$  gene results in complete loss of peripheral lymph nodes (De Togni et al., 1994).

A unique feature of this family of ligands is the ability of some members to induce directly the apoptotic death of chronically activated T cells and B cells (Daniel and Krammer, 1994; Alderson et al., 1995). Likewise, TNF has been shown to induce apoptosis in normal thymocytes under appropriate conditions (Hernandez-Caselles and Stutman, 1993). Further, peripheral T cells from HIV-infected individuals have been shown to be much more sensitive to Fas-mediated apoptosis than uninfected controls (Katsikis et al., 1995).

Because of their fundamental roles in immune and developmental networks, and particularly the involvement in programmed cell death, we have searched the expressed sequence tag (EST) library using homology to a consensus amino acid sequence based upon the portion of the  $\beta$ -pleated sheet that is most conserved across known TNF ligand family members (see Discussion). This screen identified an EST potentially encoding a new member of this family. Full-length human and murine cDNAs were subsequently cloned and sequenced, and the sequence confirmed an open reading frame predicted to encode a type II membrane protein with significant homology to the TNF ligand family. Biological studies using either the cell-bound ligand or an engineered soluble form demonstrated its ability to induce apoptosis in a wide variety of transformed cell lines.

### Results

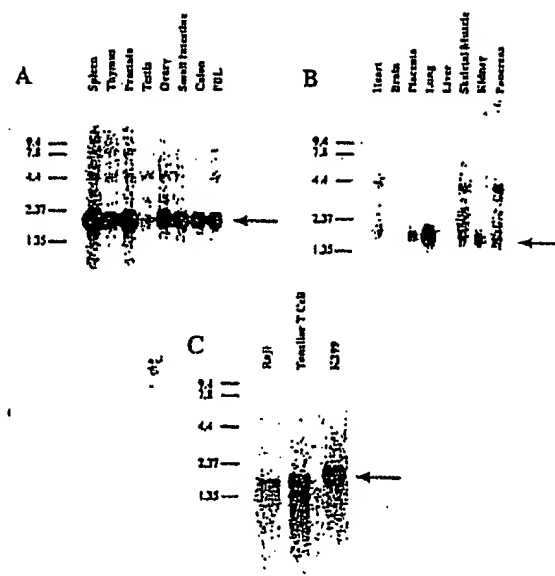
#### Isolation of Human and Murine cDNAs Encoding TRAIL

A BLAST search of the National Center for Biotechnology Information dbEST data base using a consensus amino

We have found that TRAIL, expressed on the surface of transfected cells as well as a soluble form discussed in the next section, induces apoptosis in a variety of cell lines. Fixed CV1/EBNA cells transfected with a vector express-

The cDNA sequence of the human TRAIL gene is shown. Numbers at the right indicate the nucleotide number (top) and amino acid number (bottom). Amino acids comprising the predicted transmembrane region are underlined. Underlined nucleotides near the 3' end form a consensus polyadenylation signal.

ing the full-length surface-bound form of TRAIL (pDC409-TRAIL) were examined for their ability to induce apoptotic death in target cells utilizing a DNA fragmentation assay that was performed after a 4 hr coculture of these fixed cells with either Jurkat or U937 cells (1:4 ratio of effector to target cells). As a positive control, we used soluble recombinant Fas ligand, which is known to induce apoptosis in Jurkat cells. Fragmented DNA in the cellular cytoplasm was recovered and resolved by agarose gel electrophoresis. The results demonstrate that, like conditioned supernatant from cells expressing Fas ligand, fixed cells expressing TRAIL induce DNA laddering in both Jurkat and U937 cells (Figure 3). This fragmentation of cellular DNA into soluble multimers of ~180 bp is a hallmark of apoptosis. The monoclonal antibody to human Fas (M3) has previously been shown to block activity of Fas ligand (Ramsdell et al., 1994). Consistent with this result, DNA laddering induced by Fas ligand is significantly reduced



**Figure 2. Northern Blot Analysis of TRAIL Transcripts**

RNAs isolated from the indicated tissues were resolved on a formaldehyde-agarose gel, blotted onto a positively charged nylon membrane, and probed with labeled antisense TRAIL-encoding RNA. Positions of RNA size markers in kb are shown on the left. Shown are blots using RNA from whole tissues (A,B) or cell lines and purified cells (C). All lanes used 2  $\mu$ g poly(A)<sup>+</sup> RNA, except for the K299 lane, which used 5  $\mu$ g of total RNA.

by pretreatment of the Jurkat cells with M3. The apoptotic death of the Jurkat cells induced by TRAIL was not affected by pretreatment with M3, indicating that TRAIL-induced apoptosis is not mediated through Fas.

#### Characterization of Soluble TRAIL

To facilitate biological studies, we constructed an epitope-tagged soluble form of TRAIL. Based upon published reports that state that the C-terminal conserved extracellular region of TNF is sufficient for biological activity, and that deletions of N-terminal residues can increase activity (Creasey et al., 1987), it seemed probable that the homologous C-terminal domain of TRAIL would be sufficient to produce biologically active protein. Soluble TRAIL-expressing vector was made by fusing in-frame DNA encoding the following amino acid sequences (listed from 5' to 3', respectively): a leader sequence from human cytomegalovirus (CMV), a synthetic antibody epitope (Flag), and amino acids 95–281 of human TRAIL. As shown in Figure 4, the soluble recombinant TRAIL expressed in CV1/EBNA cells has an apparent molecular weight of 28 kDa by SDS-PAGE. Gel filtration analysis of the purified soluble TRAIL suggests that the native molecule is multimeric in solution with a size of ~80 kDa (data not shown). A similar soluble form of murine TRAIL has also been expressed (data not shown).

#### Biological Activity of Soluble TRAIL

To investigate the activity of soluble TRAIL, Jurkat cells were treated with either conditioned supernatant from cells transfected with the soluble TRAIL construct, Fas

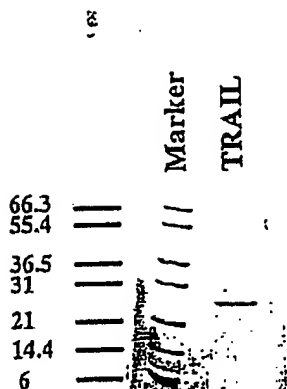
| Lane                   | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------------------------|---|---|---|---|---|---|---|---|---|---|
| Fixed CV1/EBNA         |   | + | - | + | - | - | - | + | - | - |
| Fixed CV1/EBNA-TRAIL   |   | - | + | - | + | - | + | - | + | - |
| Jurkat                 |   | - | - | + | + | + | + | + | - | - |
| Fas Ligand             |   | - | - | - | - | + | + | + | - | - |
| $\alpha$ -Fas antibody |   | - | - | - | - | - | - | + | + | - |
| U937                   |   | - | - | - | - | - | - | - | + | + |



**Figure 3. Apoptotic DNA Laddering Induced by TRAIL**

Jurkat or U937 cells were cultured for 4 hr with fixed cells or soluble factors as indicated, then fragmented DNA in the cytoplasm was recovered and resolved by 1.5% agarose gel electrophoresis. Fixed cells were obtained by transfecting CV1/EBNA cells with vector alone or TRAIL-expressing vector, and treating the cells with 1% paraformaldehyde in PBS. Fas ligand was obtained from supernatants of transiently expressing COS cells. Blocking  $\alpha$ -Fas antibody used is a monoclonal antibody, M3. The size marker in lane M is  $\phi$ 174 DNA digested with HaeIII.

ligand, or empty vector. The viability of target cells after incubation with 10  $\mu$ l of conditioned supernatants for 20 hr was monitored by metabolic conversion of the dye alamar blue. The results, shown in Figure 5A, demonstrate that



**Figure 4.** Coomassie stained SDS-PAGE of Purified Soluble TRAIL. Supernatants from CV1/EbNA cells transfected with pDC409-Flag-TRAIL were applied to an  $\alpha$ -Flag antibody column. Soluble TRAIL protein was eluted with citrate, neutralized with Tris, and resolved by SDS-PAGE. Sizes in kilodaltons of molecular mass markers are indicated on the left.

while both Fas ligand and TRAIL kill Jurkat cells, only killing by Fas ligand can be inhibited by addition of blocking antibody to Fas. Furthermore, killing by soluble TRAIL, which has been constructed with an artificial Flag epitope, can be enhanced by immobilizing anti-Flag antibody to the plate prior to adding TRAIL conditioned supernatant. This presumably facilitates cross-linking of the ligand-receptor complexes, thereby increasing the strength of signaling.

Since the process of apoptosis rapidly induces dramatic effects on cellular structure, the effects of TRAIL on Jurkat cells were visualized by confocal microscopy. Jurkat cells were treated with purified TRAIL or with an agonist antibody to human Fas receptor (CH11) for 2.5 hr (Yonehara et al., 1989). The cells were stained with fluorescence-conjugated reporter dyes, then imaged by fluorescence-scanning confocal microscopy. The results indicate that TRAIL induces blebbing of the cellular membrane and release of apoptotic vesicles that is indistinguishable from the effect of cross-linking antibody to Fas (Figure 5B, panels A-C). Green fluorescence of cellular cytoplasm is due to activation of calcein AM by a cytoplasmic esterase. Also similar to the effect of antibody to Fas, TRAIL induces the rapid destruction of the cytoskeleton and breakdown of nuclear integrity (Figure 5B, panels D-F). In these panels, F-actin appears green due to staining with a BODIPY FL-conjugated phalloidin, and nuclei appear red due to staining with propidium iodide. These changes in cellular morphology are indicative of apoptosis (Wyllie et al., 1982).

Since TRAIL was found to induce apoptosis in Jurkat and U937 cells, the effect of TRAIL on various other cell lines was also tested. Cell lines indicated in Table 1 were assayed by incubation for 20 hr with conditioned supernatants from pDC409-Flag-TRAIL transfected CV1/EbNA cells versus supernatants from cells transfected with empty vector, in the presence of immobilized anti-flag anti-

body. Metabolic activity was assayed by conversion of alamar blue dye. As shown in Table 1, many cell lines of hematopoietic origin are sensitive to TRAIL-mediated killing. True cell death (apoptotic in nature) was confirmed by trypan blue staining and visualization of apoptotic fragmentation by microscopy (data not shown). In addition, some cell lines were not killed by TRAIL, indicating that TRAIL-induced apoptosis is target cell specific.

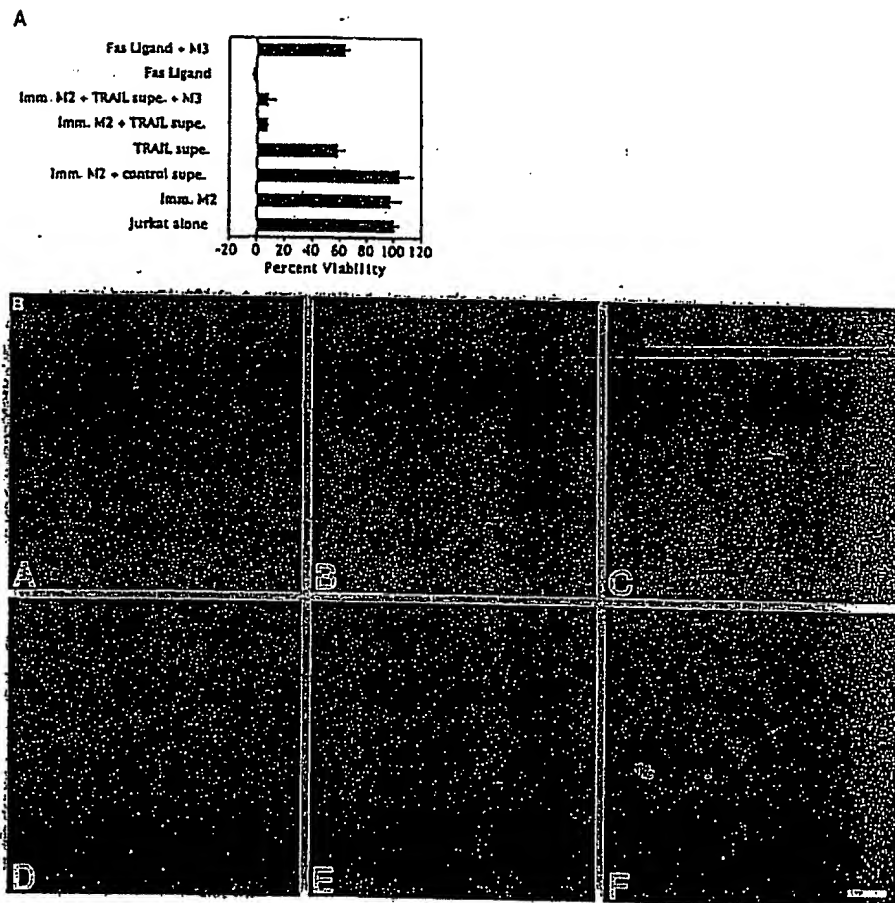
Interspecies cross-reactivity of human and murine TRAIL was tested by incubating murine TRAIL with the human melanoma line A375. Since this is an adherent cell line, the crystal violet assay, rather than alamar blue, was used to determine viability. The results demonstrate that both human and murine TRAIL are active on these human cells (Figure 6A). Conversely, to test the ability of human TRAIL to act on murine cells, we utilized the murine fibroblast cell line L929. As shown in Figure 6B, incubation of L929 cells with either human or murine TRAIL results in a decrease in crystal violet staining, thus demonstrating that human TRAIL is also active on murine cells. In addition to crystal violet, cell death was confirmed by trypan blue staining (data not shown).

#### Chromosomal Mapping of TRAIL

To determine where the TRAIL gene resides in the human genome, metaphase chromosomes from two normal males were analyzed by fluorescent in situ hybridization. From one male, 20 metaphases were examined for fluorescent signal. All of these metaphases showed signal on one or both chromatids of chromosome 3 in the region 3q25-q26.3; 64% of this signal was located at bands 3q26.1 and 3q26.2 (data not shown). There was a total of 10 nonspecific background dots observed in these 20 metaphases. A similar result was obtained from hybridization of the probe to 18 metaphases from a second normal male (data not shown). This indicates that the TRAIL gene is located in band 3q26 most likely in the region q26.1-q26.2.

#### Discussion

In this paper, we have described the molecular cloning and biological characterization of a novel member of the TNF ligand family. Like all but one other member of this family, the predicted TRAIL protein product has the characteristics of a type II membrane protein, i.e., no leader sequence, and an internal transmembrane domain. Also like other TNF family ligands, TRAIL has an N-terminal (cytoplasmic) domain, which is not conserved across family members, while the C-terminal (extracellular) domains show significant conservation (Smith et al., 1994). The percent identity of the human extracellular C-terminal domain of TRAIL to the most closely related members of the TNF ligand family, Fas ligand, TNF $\alpha$ , and LTP, is 28%, 23%, 23%, and 22%, respectively. Alignment of the C-terminal amino acid sequences of these related family members with human and murine TRAIL is shown in Figure 7. The crystal structures of TNF and LT $\alpha$  are known and these ligands have been shown to fold into  $\beta$ -pleated



**Figure 5. Death of Jurkat Cells Induced by Soluble TRAIL.**

Jurkat cells were cultured for 20 hr in the presence or absence of TRAIL under the indicated conditions. Metabolic activity was assayed by conversion of stamper blue dye and plotted as a percentage relative to control cultures (A). Shown are confocal microscopic images of Jurkat cells in the early stages of apoptosis (B). Cells were treated with medium only (A and D), 20 ng/ml purified TRAIL in the presence of immobilized M2 antibody to Flag (B and E), or cross-linking antibody to Fas receptor (C and F) for 2.5 hr. The live/dead cell assay used in (A-C) employs a dye (calcein AM) that fluoresces green in response to intracellular esterase activity, revealing blebbing of the cellular membrane and release of apoptotic vesicles. Cells in (D-F) are stained with BODIPY FL phalloidin (green), showing a pronounced disruption of the cytoskeleton. The nuclei are stained with propidium iodide (red). The solid white bar in (F) indicates a length of 10  $\mu$ m.

sheet sandwich structures and to form homotrimers (Jones et al., 1988; Eck et al., 1992). The sequences in this region that are most conserved map to the strands that form these  $\beta$ -pleated sheets, with the centrally located D strand having the greatest conservation. Therefore, it is likely that TRAIL, like TNF, forms an oligomeric structure that is necessary to cross-link its cognate receptor, thereby transducing a signal to the target cell.

In contrast with the extracellular region, the N-terminal

cytoplasmic domains of previously identified TNF ligand family members are conserved across species, though not between family members. This fact, combined with data demonstrating that cross-linking of some of these molecules can directly induce biological effects, suggests that some of these ligands may themselves transmit signals across the cell membrane (Cayabyab et al., 1994; Potluk et al., 1994; Süßer et al., 1995). However, the short cytoplasmic domain of TRAIL is not conserved between

Table 1. Effect of Soluble TRAIL on Cell Line Viability

| Cell Line | Description                    | Percent Viability* |
|-----------|--------------------------------|--------------------|
| Bjab      | Burkitt lymphoma               | 0.5 ± 3.8          |
| Ramos     | Burkitt lymphoma               | 12.1 ± 2.1         |
| U937      | Histiocytic lymphoma           | 25.2 ± 8.2         |
| HL60      | Promyelocytic leukemia         | 59.5 ± 3.2         |
| Raji      | Burkitt lymphoma               | 64.9 ± 4.5         |
| Dauid     | Burkitt lymphoma               | 70.2 ± 4.2         |
| THP-1     | Monocytic cell line            | 92.3 ± 8.8         |
| K562      | Chronic myelogenous leukemia   | 97.1 ± 4.8         |
| K299      | Large cell anaplastic lymphoma | 99.0 ± 4.3         |
| MP-1      | Spontaneous B cell line        | 104.9 ± 11.7       |

\*Results are means ± SEMs of four wells for each data point.

species, suggesting that it is unlikely that this domain of TRAIL serves a biological function.

Known biological activity of TRAIL is thus far limited to inducing apoptosis in various cell lines. Including but not restricted to those of hematopoietic origin. Apoptosis, the process of programmed cell death, is necessary for the normal development and homeostasis of an organism, and involves dramatic changes in cellular structure. TRAIL has been shown in this paper to induce many of these changes such as blebbing of the cellular membrane, disruption of the cytoskeleton, fragmentation of DNA into ~180 bp multimers, and decimation of metabolic activity (Figures 3, 5). While some members of the ligand family, such as TNF and LTα, can induce apoptosis in certain cell lines, of the known family members, only Fas ligand and TRAIL have the ability to kill such a wide variety of cell lines. Intriguingly, of the known TNF ligand family members, the primary sequence of TRAIL is most closely related to Fas ligand.

The *in vivo* role of Fas or Fas ligand in apoptosis has been shown by spontaneous mutations in mice (Watanabe-Fukunaga et al., 1992; Lynch et al., 1994; Takahashi et al., 1994) and in humans (Fisher et al., 1995; Rieux-Laucat et al., 1995), which result in lymphadenopathy and development auto-immune disease. Unfortunately, insight into the *in vivo* role of TRAIL is not offered by its location on chromosome 3, since, at this time, no human disease with a similar phenotype has a locus that has been mapped nearby. Of course, this does not eliminate the potential role of TRAIL in disease, since many human diseases have not yet been mapped. Also, it has previously been observed that some of the TNF-related genes are clustered in the genome. TNF, LTα, and LTβ are tightly linked on chromosome 6, while CD27 ligand and 4-1BB ligand both map to chromosome 19p13.3 (Browning et al., 1993; Goodwin et al., 1993b). In contrast, TRAIL does not map near any of the known TNF ligand family members. This does not preclude, of course, clustering with other TNF family members that are as yet unidentified.

One significant difference between TRAIL and Fas ligand is the tissue distribution of their transcripts. Unlike Fas ligand, whose transcripts appear to be largely restricted to stimulated T cells (Suda et al., 1993, 1995) (Figure 2), significant levels of TRAIL are seen in many tissues, and it is constitutively transcribed by some cell lines.

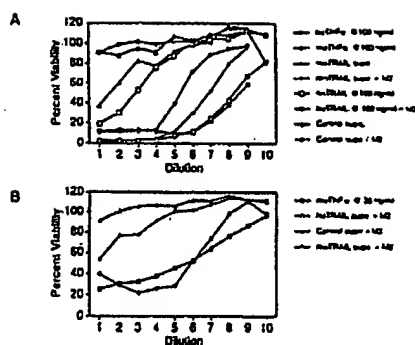


Figure 6. Cross-Species Activity of Human and Murine TRAIL. Cells were incubated with various factors as indicated and viability was assayed by staining with crystal violet dye and plotted as a percentage of control cultures. Starting concentrations of factors are shown in the legend, and the horizontal axis indicates the number of serial 2-fold dilutions of these factors. The effect of human and murine soluble TRAIL on human A375 cell viability is plotted (A). The effect of human and murine soluble TRAIL on murine L829 cell viability is plotted (B).

Therefore, TRAIL must not be cytotoxic to most tissues *in vivo*. However, it is interesting to note that TRAIL transcripts are not found in the liver, the same organ that is destroyed by *in vivo* injection of antibody to the Fas receptor (Watanabe-Fukunaga et al., 1992). TRAIL transcripts are present in the thymus, whereas Fas ligand is not (Suda et al., 1995) (Figure 2). Furthermore, unlike Fas ligand, TRAIL, acting as a single agent, does not kill freshly isolated murine thymocytes (Ogasawara et al., 1995; H. J. McKenna, unpublished data). This does not rule out a role for TRAIL in deletion of inappropriate T cells in the thymus, but if TRAIL is involved in thymic selection, additional signals are required to induce apoptosis. Also, TRAIL acting as a single agent does not induce apoptosis in primary B cells or T cells (data not shown), and TRAIL has not been able to costimulate primary T cells to proliferate in the presence of suboptimal amounts of anti-CD3 (data not shown).

Given the rather widespread expression of TRAIL and its ability to induce apoptosis in so many different types of cultured cells, it is reasonable to infer that either the TRAIL receptor is restricted in its distribution, or that it acts to induce apoptosis only under certain restricted circumstances. Interaction of known TNF receptor family members and TRAIL was tested using a very sensitive radio-binding assay. Labeled fusion proteins of the extracellular domains of TNF receptor family members and immunoglobulin Fc were used to probe CV1/EBNA cells transfected with either pDC409-TRAIL or a similar vector expressing the known cognate ligand. While strong signals were seen in the cells expressing the known ligands, no signal above background was seen with the TRAIL-expressing cells, indicating that no known member of the TNF receptor family binds to TRAIL (data not shown).

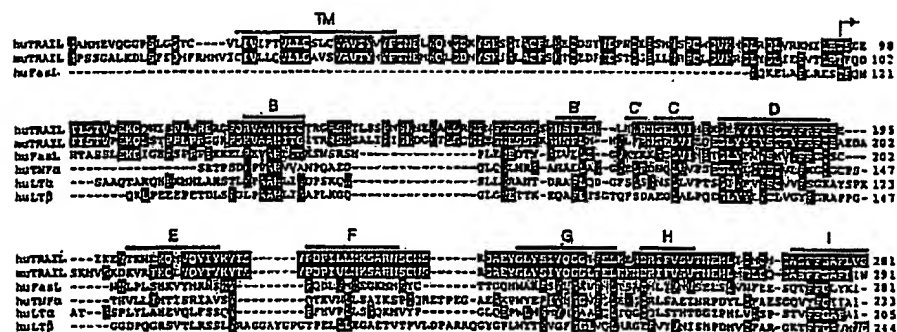


Figure 7. TRAIL Sequence Homologies

Human and murine TRAIL predicted amino acid sequences were aligned with other TNF ligand family members. The entire predicted amino acid sequence of the human and murine TRAIL genes are aligned, and alignments of other ligands start where significant homology begins. Solid background shows identity with at least one aligned residue in another family member, or between the murine and human sequences. Solid bars marked with letters A-I indicate  $\beta$  strands in the TNF $\alpha$  crystal structure. The shaded bar marked TM indicates the transmembrane region. The arrow indicates the N-terminus of the coding regions used to make soluble versions of the human and murine ligands.

These data are corroborated by the failure of blocking antibody to Fas receptor to prevent TRAIL-induced apoptosis (Figures 4 and 5), and the ability of Fas ligand to induce apoptosis in primary thymocytes whereas TRAIL does not (Ogasawara et al., 1995; H. J. McKenna, unpublished data). Therefore, it is likely that the receptor for TRAIL is a novel member of the TNF $\alpha$  receptor family, which, when identified, should provide valuable insight into the biological function of TRAIL.

#### Experimental Procedures

##### Cells

The MP-1 line is a spontaneously derived Epstein-Barr virus-transformed B cell line (Goodwin et al., 1983a). K299 (DSM-ACC31) was established from peripheral blood of a male diagnosed with high grade LCAL (Fischer et al., 1988). Other cell lines referred to in this paper have been deposited in and described by the American Type Culture Collection (Rockville, Maryland). A375 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100  $\mu$ g/ml streptomycin, and 100  $\mu$ g/ml penicillin. All other cell lines were cultured to a density of 200,000-500,000 cells per ml in RPMI medium supplemented with 10% fetal bovine serum, 100  $\mu$ g/ml streptomycin, and 100  $\mu$ g/ml penicillin.

##### Cloning of the TRAIL cDNA

An EST that potentially encodes a novel TNF ligand family member was identified in the dbEST data base at the National Center for Biotechnology Information by performing a TBLASTN search using the query sequence LVVXXGLYVYXXQVAF (GenBank accession number Z35728). This sequence is based upon the most conserved region of the TNF ligand family, the D strand. This EST sequence was obtained from a heart atrium cDNA library submitted by Genzentrum Muenchen, Laboratorium für molekulare Biologie (Kloppspitz 18a, 82152 Martinsried, Federal Republic of Germany).

Oligonucleotides (30 bp) were synthesized based upon the sequences from the 5' and 3' ends of this EST file: TGACGAGAGAGATATGACAGCCCTGCTG and TGAATTCGAAAGTATGTTTGGAATAGATG, respectively. Of each oligonucleotide, 50 ng were labeled with [ $\gamma$ -<sup>32</sup>P]ATP and polynucleotide kinase. 1  $\mu$ g cDNA libraries (2) were screened by conventional methods (Sambrook et al., 1989) using an equimolar mixture of these labeled oligonucleotides as probe. One library was a human heart 5' stretch cDNA library (Stratagene), and

the other was a human PBL library that has been previously described (Idzarda et al., 1990). Nitrocellulose filters were lifted from these plates in duplicate, and hybridized overnight with the kinased oligonucleotides at 67°C in a solution of 60 mM Tris (pH 8.0), 2 mM EDTA, 5 x Denhardt's solution, 8 x SSC, 1 mg/ml n-lauryl sarcosine, 0.5% NP40, and 4  $\mu$ g/ml denatured salmon sperm DNA. The filters were then washed in 3 x SSC at 67°C for 30 min. One positive plaque was obtained from approximately 10<sup>6</sup> plaques using a heart 5' stretch cDNA library (Stratagene). Approximately 50 positive plaques were obtained out of 5 x 10<sup>6</sup> plaques using the PBL library. Of these first round positive plaques, 15 were picked and the inserts from the enriched pools were amplified using oligonucleotide primers designed to amplify phage inserts. The resulting products were resolved by 1.5% agarose gel electrophoresis, blotted onto nitrocellulose, and analyzed by standard Southern blot techniques using the two kinased EST oligonucleotides as probes. The two plaque picks that produced the largest bands by the Southern blot analysis were used for secondary screening, and isolated phage plaques were obtained using the same procedures as described above. DNA from the isolated phage were prepared by the phage lysis method, and the cDNA inserts were excised with EcoRI, and ligated into the pBluescript SK(+) plasmid (Stratagene). These inserts were then sequenced by conventional methods, and the resulting sequences were aligned.

A previously described cDNA library (Mosley et al., 1985) prepared from the murine helper T cell line 7B9 in  $\lambda$  Zap vector (Stratagene) was screened with a random primed probe of a 843 bp polymerase chain reaction (PCR) product of the human TRAIL cDNA, which encompassed the TRAIL coding region. Nitrocellulose filter lifts were hybridized as previously described (Mosley et al., 1985), except that the temperature was lowered to 37°C. The filter lifts were then washed at 50°C in 1 x SSC, 0.1% SDS. Positive clones were purified and the inserts excised as pBluescript clones using helper phage as described by the manufacturer.

##### DNA

Oligonucleotides were synthesized corresponding to the 5' and 3' ends of the coding regions of the human TRAIL gene with SalI and NotI restriction sites appended to the ends of the oligonucleotides. The sequence of the 5' and 3' oligonucleotides, respectively, are as follows: GCACGTCGACACAGGATCATGGCTATGATGG and CGTGAAGCGGCSCCAGGTGAGTTAGCCCACT.

The coding region of the human TRAIL gene was amplified by standard PCR techniques, cut with SalI and NotI, and inserted into the SalI and NotI sites of the pDC409 mammalian expression vector (Smith



et al., 1993) or the pBluescript SK(+) (Stratagene) vector to create pDC409-TRAIL and pBluescript-TRAIL, respectively.

pDC409-Flag-TRAIL was created by PCR amplification of TRAIL cDNA encoding amino acids 85-281. The 3' oligonucleotide was the same as used to create pDC409-TRAIL and the 5' oligonucleotide was GTCACTAGTTCTGACTACAAGGACGACGATGACAAGACCTCTGAGGAACCATTTT, which adds a SpeI site and synthetic Flag epitope encoding region to the 5' and (Hopp et al., 1988). The resulting PCR product was cut with SpeI and NotI, and inserted into SalI and NotI cut pDC409 along with annealed oligonucleotides encoding a putative CMV open reading frame leader (Rawlinson and Barrell, 1992). This produced an open reading frame encoding the CMV leader, the Flag epitope, and human TRAIL amino acids 85-281. A parallel construct, pDC409-Flag-muTRAIL, was created in the same way but using a PCR fragment generated from the murine TRAIL cDNA using the 5' and 3' PCR primers GCGTCACCTAGTTCTGACTACAAGGACGACGATGACAAGACCTTTTCAAGGACCATTTT and ATAGCGGCCGCTGTGTTTATCTTTACTTGCT, respectively.

#### Purification of Soluble TRAIL

Supernatants from CV1/EBNA cells were harvested 3 days after transfection with pDC409-Flag-TRAIL. These were applied to a column containing the M2 anti-Flag antibody (Hopp et al., 1988), immobilized to a solid support, and washed with PBS. Fractions (800 ml) were eluted with 50 mM citrate and immediately neutralized in 0.45 M Tris (pH 8). Fractions were adjusted to 10% glycerol and stored at -20°C until needed.

#### RNA Hybridization

Northern blot analysis of RNA samples was performed by using Clontech (Palo Alto, California) multiple tissue Northern blots I and II, or by resolving RNA samples on a 1.1% agarose-formaldehyde gel and blotting onto Hybond-N as recommended by the manufacturer (Amersham Corporation), and staining with methylene blue to monitor RNA concentrations. Antisense RNA probe was generated using T3 RNA polymerase and pBluescript-TRAIL linearized with SalI as template.

#### DNA Laddering Apoptosis Assay

CV1/EBNA cells grown in Falcon T175 flasks were transfected with 15 µg of either pDC409 or pDC409-TRAIL vector. These cells were then cultured for 3 days at 37°C and 10% CO<sub>2</sub>, then fixed as previously described (Smith et al., 1993). Of these cells, 4 × 10<sup>6</sup> per well were cocultured in a 6-well plate with 2.5 ml of medium with the indicated combinations of fixed cells or concentrated supernatants from COS cells transfected with Fas ligand. Indicated cells were pretreated for 10 min with 10 µg/ml of M3, a monoclonal antibody to Fas receptor (Ramadas et al., 1994; Alderson et al., 1995). After incubation for 4 hr at 37°C and 10% CO<sub>2</sub>, fragmented DNA in the cytoplasm was recovered as described (Ishida et al., 1992), except the cell lysates were extracted three times with 1 ml of 25:24:1 phenol-chloroform-isomyl alcohol, and ethanol precipitated in the presence of 5 µg of glycogen carrier.

#### Percent Viability Assay

Cells were incubated with the indicated factors in 96-well plates in a volume of 100 µl, and assayed by alamar blue (Figure 5A; Table 1) or crystal violet (Figure 6). All cells were cultured at 5 × 10<sup>5</sup> cells per well except for A375 cells, which were cultured at 1 × 10<sup>5</sup> cells per well. Where indicated, immobilized anti-Flag antibody, M2, was added at a concentration of 10 µg/ml in a volume of 100 µl per well (Hopp et al., 1988) and allowed to adhere either overnight at 4°C or 2 hr at 37°C, then aspirated and washed twice with PBS. The incubation period was 72 hr for all cells, except the A375 cells, which were incubated for 72 hr. Conditioned supernatants were used at a concentration of 10 µl per well. Alamar blue conversion was measured by adding 10 µl of alamar blue dye (Biosource International, Camarillo, California) per well, and subtracting the OD at 550-600 nm at the time the dye was added from the OD 550-600 nm after 4 hr. No conversion of dye is plotted as 0% viability, and the level of dye conversion in the absence of TRAIL is plotted as 100% viability. When shown, error bars represent the standard deviation of measurements from four independent

wells, and the values are the average of these measurements. Crystal violet staining was performed as described (Flick and Gifford, 1984). Percent viability is calculated by multiplying the ratio staining of experimental versus control cultures by 100. To confirm that the changes in dye activities of both the alamar blue and crystal violet assay were due to cell death, the decrease in cell viability induced by TRAIL was confirmed by staining the cells with trypan blue.

#### Confocal Microscopy

Live/dead viability cytotoxicity assays were performed as recommended by the manufacturer (Molecular Probes, Incorporated, Eugene, Oregon). BODIPY FL phalloidin and propidium iodide were purchased from Molecular Probes. Cells were stained with these reagents and visualized using a confocal laser scanning microscope as recommended by the microscope manufacturer (Molecular Dynamics, Sunnyvale, California).

#### Chromosomal Mapping

The human TRAIL coding region was nick-translated with biotin-14-dATP and hybridized in situ at a final concentration of 20 ng/ml to metaphases from normal males. The fluorescence in situ hybridization method was modified from that previously described (Callen et al., 1990), in that chromosomes were stained before analysis with both propidium iodide (as counterstain) and DAPI (for chromosome identification). Images of metaphase preparations were captured by CCD camera and computer enhanced.

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#### Genbank Accession Numbers

The accession numbers for the sequences reported in this paper are U37518 for human TRAIL and U37522 for murine TRAIL.